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ApoE in Oral Lichen Planus

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Abstract

Background: Oral Lichen Planus is a common chronic mucocutaneous disease of unknown etiology. It affects up to 3% of females, is four times less frequent in males and is diagnosed predominantly after the age of 40.

Aim: The goal of this study was to investigate whether ApoE was upregulated in OLP and whether increased ApoE could differentiate human monocytic cell lines to dendritic cells.

Method: Gene expression from two patients diagnosed with OLP was analyzed using microarray. Selected genes were then analyzed using RT-PCR to confirm the results. Multicolor immunohistofluorescence staining for ApoE expressing macrophages were performed on cryosections from on OLP lesions. Two human monocytic cell lines (U937, THP-1) was incubated with a humanized version of previously reported self peptide (Ep1.B) derived from murine ApoE to test for dendritic cell maturation.

Results: Microarray based expression studies revealed that mRNA for ApoE was 8-14 times more expressed in diseased OLP lesions compared to less diseased areas from the same patient. RT-PCR for ApoE and CD3 revealed that the ApoE gene expression level correlated to T cell infiltration (CD3delta) and thus to the inflammation. Immunohistochemistry did not reveal any strong macrophage specific ApoE expression, in contrast to CD68+ macrophages in the tonsils. Stimulation of the human monocyte cell lines with a humanized version of the ApoE derived self peptide revealed a strong protein agglutinating property of the peptide, making cellular analysis almost impossible. However, the humanized self-peptide induced, apparently an increased translocation of CD205 (DEC-205) from cytoplasm to the cellular membrane. Thus, the self-peptide may induce monocytes to become more functional dendritic cells, but further analysis could not be performed due to the peptides ability to agglutinate both cells and serum proteins in the culture media.

Conclusion: Although murine ApoE may play a role in immune regulation, there is as of now no evidence to support a conclusion that human ApoE play a key role in the complex immunopathology of oral lichen planus.

Abbreviations

OLP: Oral Lichen Planus

DC: Dendritic Cell

Treg: Regulatory T-lymphocytes

Th-1: T helper cell 1

Th-2: T helper cell 2

Th-17: T helper cell 17

IHC: Immunohistochemistry

GARP: a cell surface protein specifically expressed on Treg post–TCR activation

CD11c: Dendritic cell marker

DEC205: Dendritic cell marker.

FoxP3: Regulatory T-cell marker

CD25: T-cell marker

CD86: Dendritic cell marker, necessary for T cell activation and survival.

Rt-PCR: Real Time Polymerase Chain reaction

Microarray: Method for detecting and quantifying large numbers of genes simultaneously

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Also a big thank you to principal engineer Solveig Stig for technical support, patience and help.

Introduction – subject selection and motivation

Oral Lichen Planus (OLP) is a common chronic bilateral mucocutaneous disease of unknown etiology. It affects up to 3% of females, is four times less frequent in males, is diagnosed predominantly after the age of 40 and may present itself as one of six clinically defined forms (8).

Reticular OLP is the most common form. The typical lesion is on the buccal surface which is covered with numerous interlacing white keratotic lines or striae (Wickhams striae). This form is usually asymptomatic and discovered during routine examination. Reticular form is often present alongside the other clinical OLP forms (25)



Figure 1: Reticular OLP on buccal mucosa. Note the erosive lesion in the center. Picture taken from www.cixip.com/index.php?pagecontentid1033)

Erosive OLP shows a painful central ulceration with a pseudo membrane covering the ulceration. It is the second most common clinical presentation after reticular form (25)



Figure 2: Erosive lichen planus on the tongue. Picture taken from oral-lichen-planus.blogspot.no

Plaque OLP resembles leukoplakia, but has a multifocal distribution, usually on dorsum of the tongue or on the buccal mucosa (25)



Figure 3: Plaque form on the lateral border of the tongue.
Picture taken from www.dentalcare.com

Erythematous/atrophic OLP present itself as red patches with very fine white striae, usually on attached gingiva. Patients complain of burning, sensitivity and general discomfort. This form may resemble cicatricial pemphigoid (25).



Figure 4: Erythematous/atrophic lichen planus on attached gingiva.
Picture taken from www.dentalcare.com

The Bullous OLP variant has short lived bullae, which leaves a painful ulceration when rupturing (25).

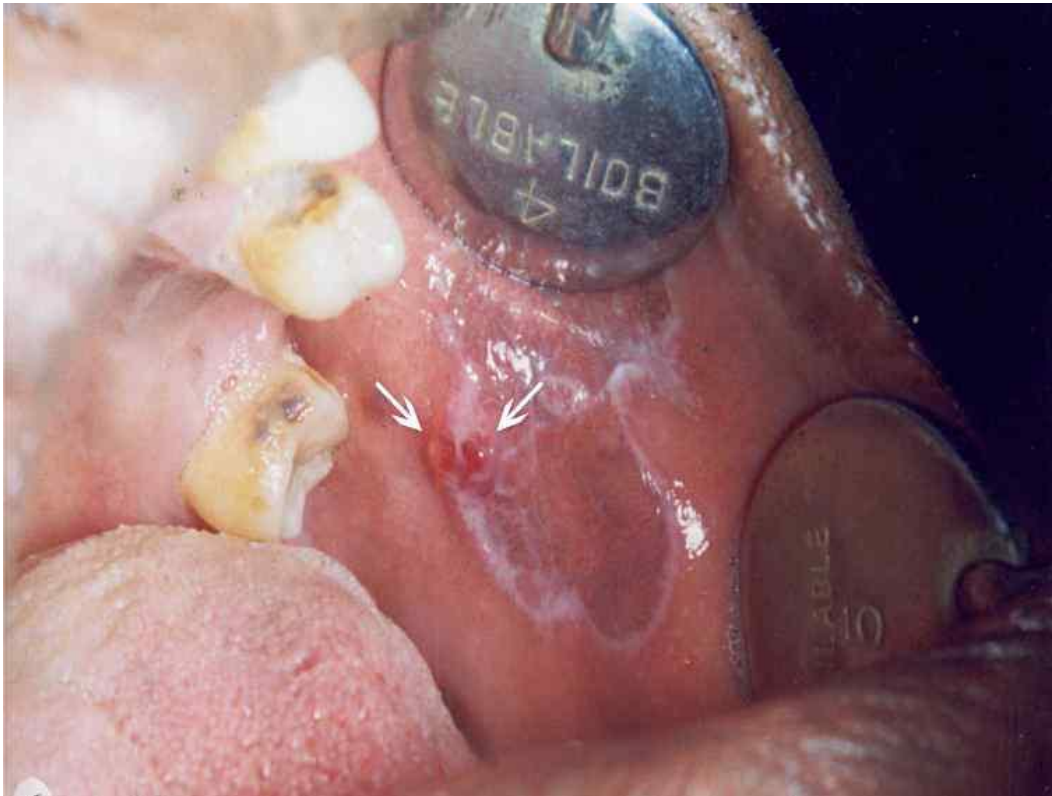


Figure 5: Bullous oral lichen planus on buccal mucosa (arrow). Bullae normally rupture quickly and leave a erosive like wound. Picture taken from screening.iarc.fr/atlasora.

Although reticular and erosive OLP's are the most common variants, they often coexist together with the other subtypes.

Treatment of OLP can be a challenge. Asymptomatic lesions can usually be observed without any treatment. However, around 70% of OLP patients report symptoms from the lesions (2). For these patients, active treatment such as locally and systemic corticosteroids, as well as surgical intervention may be considered. As many as 20-60% of OLP patients may have additional cutaneous lesions (25).

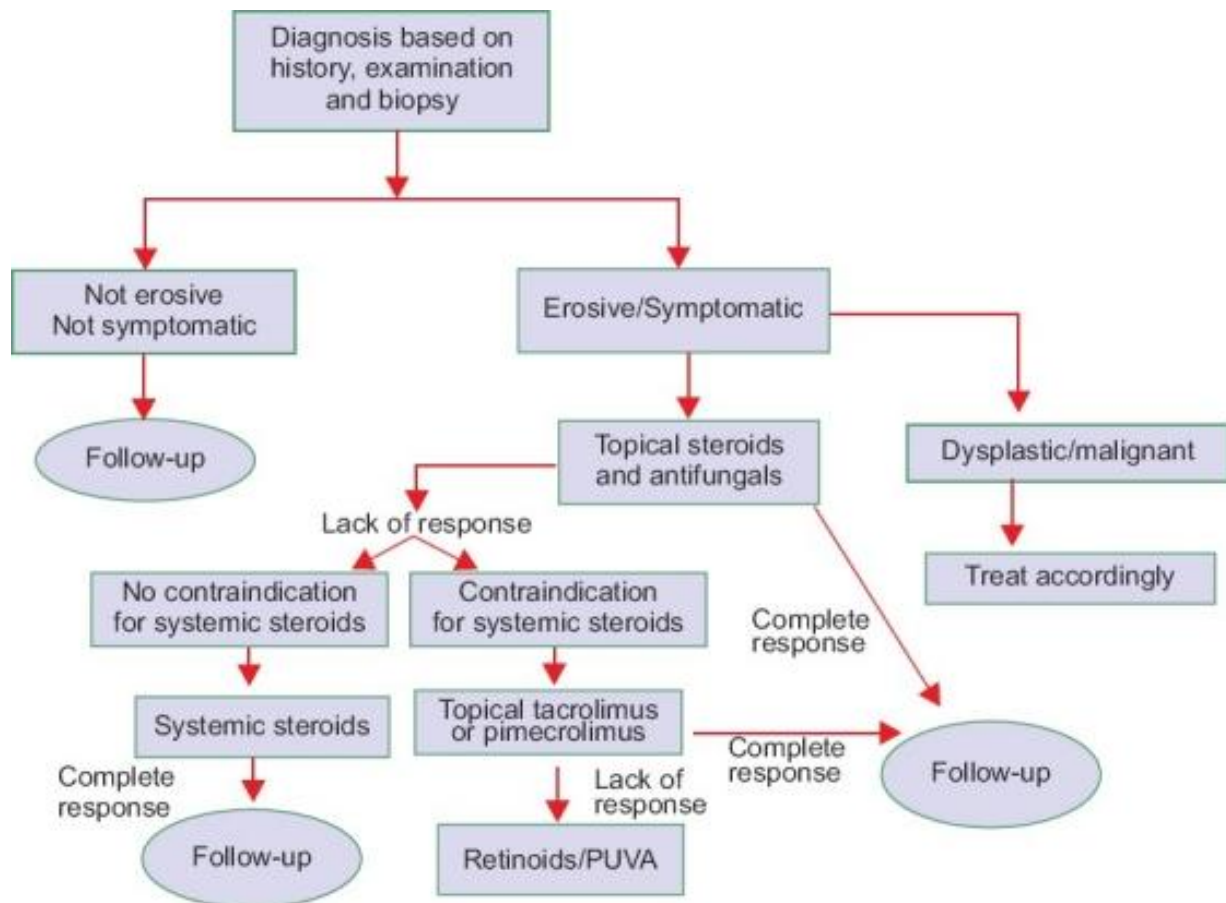


Figure 6: Flow charts showing the clinical follow up of patients with OLP (26). Follow-up interval at least every 6 months with dentist or oral surgeon. Cancer suspect lesions are referred immediately to specialist. Treatment with steroids is the first choice in patients with symptomatic lesions. Preferably administered locally, but systemic use is considered with lack of clinical effect. OLP lesions are often infected with candida species and for this reason antifungal treatment should always be considered as a supplement. Tacrolimus (FK-506 or fujimycin) is a macrolide class immunosuppressive drug commonly used in treatment of allogeneic organ transplant. It is an alternative to steroid treatment when contraindications for this treatment are present. Retinoids are a class of drugs chemically related to vitamin A. It has immunosuppressive properties and as such it can be an alternative the in treatment of OLP.

OLP is histologically characterized by an intense sub epithelial T lymphocyte infiltration and disruption of the basal membrane. The pronounced apoptosis of basal keratinocytes may be caused by CD8+ cytotoxic T-cells (3), resembling a hypersensitivity reaction.

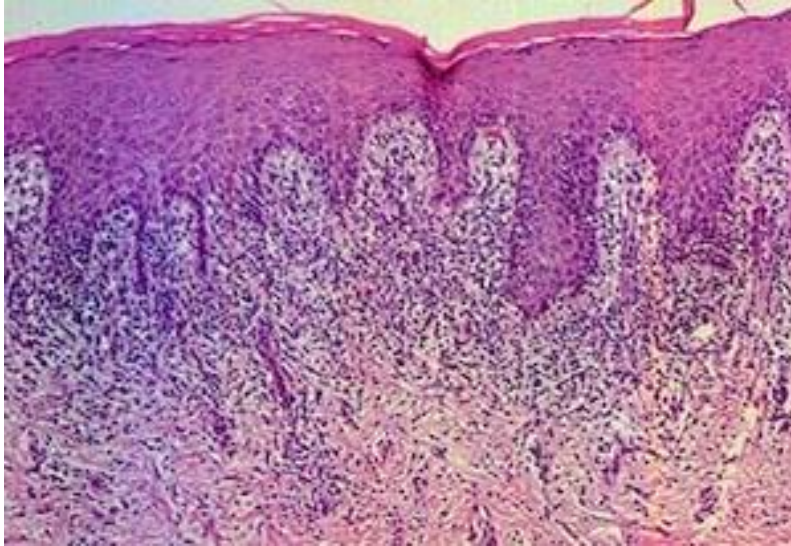


Figure 7: Histological picture of OLP. Hyperkeratosis and saw tooth shaped epithelium with intense lymphocyte infiltration underneath the basal lamina.

Eosinophilic colloid bodies along the epithelial-connective tissue interface (Civatte bodies) are frequently found. The inter papillary ridges may either be absent, hyperplastic or saw tooth-shaped. The thickness of the spinous layer varies. OLP lesions display various degrees of ortho or parakeratosis (18).

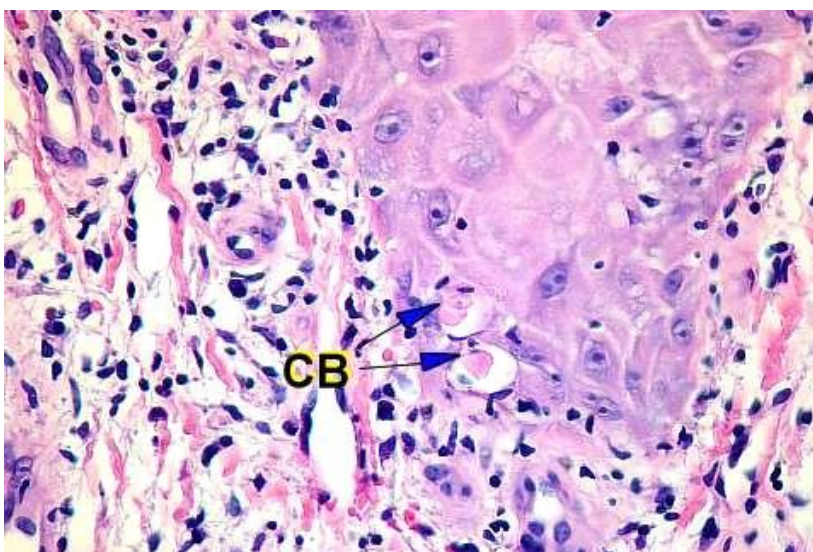


Figure 8: Civic bodies (CB) in an OLP lesion
(<http://www.flickr.com/photos/asnaan1/4159937756/sizes/m/in/photostream/>)

OLP is usually not considered lethal disease, but it may be difficult to distinguish from more serious conditions, such as leukoplakia and squamous cell carcinoma. However, the risk of malignant transformation is less than 2% (8). OLP may sometimes exacerbate in periods of psychological stress and anxiety, which may hint to dysregulation of the immune system (3).

There have been speculations that both bacterial and viral infections, autoimmunity, immunodeficiency, food allergies, stress, habits, trauma, diabetes and hypertension, malignant neoplasms and bowel diseases may increase the risk of OLP, but no definite proof has yet been put forward (26).

Of the different etiological theories, there is much debate about the autoimmune nature of OLP. Thus, an antigen-specific cell mediated immune response directed to a yet unknown, epithelial antigen(s). Some of the histological feature of OLP is similar to a chronic host versus graft disease. Presentation of antigens by basement layer keratinocytes may therefore induce cytotoxic T lymphocyte (CD8+) to kill these basal cells (18). Such reaction would be HLA-class-I dependent, whereas antigen presentation in HLA-class-II may be mediated by intraepithelial Langerhans cells (LCs) or directly by keratinocytes, both of which have increased HLA-DR expression in the lesion, presumably due to cytokines produced by the subepithelial lymphocyte infiltrate (26). As in all inflammatory reactions, there are increased expression of adhesion molecules such as ELAM-1, ICAM-1 and VCAM-1 to mediate lymphocyte homing to the OLP lesion, which may explain the massive subepithelial infiltrate in OLP and the presence of hepatitis C specific T cells in the infiltrate without any evidence for active hepatitis C production in the lesion.

There has been some speculation that the primary defect in OLP is the disruption of the basement membrane. Some of the T cells may be drawn to the lesion by the pre-existing inflammation in OLP. These non specific mechanisms may activate the innate immune system, activate metalloproteinase which disrupt the epithelial basement membrane, induce apoptosis of basal keratinocytes allowing non-specific T cells to migrate into the epithelium and to set up a chronic inflammatory reaction (25). Matrix metalloproteinase, chemokine's and mast cells all contribute to the increased degree of inflammation and may participate in the chronicity of the disease (26).

OLP has long been suspected to be an autoimmune disease and many theories have been proposed to compensate for the lack of evidence.

- 1) Lower expression of TGF-B1 has been found in OLP. TGF-B1 has immunosuppressive effects. This may predispose for the condition and also play a part in the chronicity.
- 2) Normal oral mucosa is considered an immune privileged site. A breakdown of this may contribute to the disease. In particular the balance between keratinocyte apoptosis triggered by infiltrating T cells and T cell apoptosis triggered by resident keratinocytes. Failure in the latter mechanism may cause excessive T cell infiltration in epithelium (26).
- 3) Dendritic cells and LCs mature in OLP in response to inflammatory cytokines (IL-1B, TNF-Alfa), CD40L (CD154) expressed on activated T-cells, necrotic cells, heat shock proteins, nucleotides among others (26).
- 4) Heat shock proteins are up regulated in OLP. Possibly by drugs, infections, bacterial products and trauma. This may cause T cells to proliferate (26).

Epidemiological evidences suggest that chronic Hepatitis C Virus (HCV) infection may be an etiologic factor in OLP. Although, this association is prevalent in Southern Europe, Japan and USA, it is not observed in countries with the highest HCV prevalence suggesting that other factors than HCV infection alone mediates the observed association with OLP. In OLP, HCV replication has been reported in the epithelial cells from the LP lesions by reverse transcription/polymerase chain reaction and by *in-situ* hybridization. Moreover, HCV-specific CD4 and CD8 lymphocytes could be isolated from the subepithelial band. This may either suggest that HCV-specific T lymphocytes participate in the immunopathogenesis of OLP, or that the lesion attract memory T cells in a non-specific manner (by standing phenomenon). Whether HCV infected patients have increased risk of developing OLP or patients with OLP have enhanced risk of developing HCV infection is yet to be answered (26) Studies show that patients with hepatitis C virus have a higher prevalence of OLP than control groups (2). However this apparent connection may be explained by differences in the incidence of Hepatitis C infection (23).

Differential diagnosis and lichenoid reactions (LR)

Differential diagnosis include, lupus erythematosus, leukoplakia, erythroleukoplakia, white sponge nevus, candidiasis, proliferative verrucous and leukoplakia (18).

Graft vs. Host disease (GVHD) is a common serious complication following allogeneic tissue transplant. Oral involvement occurs in 33% to 75% of patients with acute GVHD and up to 80% of patients with chronic GVHD (3). Oral mucosal GVHD resembles OLP both clinically and histologically.



Figure 10: Lichenoid reaction on the buccal mucosa in a patient with graft vs host disease. From www.ispub.com

Oral mucosal lichenoid lesions may also develop after systemic drug administration. Lichenoid drug reactions (LDR) may be unilateral but usually appear bilateral, similar as idiopathic OLP. Drugs that have been implicated in oral LDR include NSAIDs, ACE inhibitors and beta-blockers.

Oral mucosal lichenoid lesions (LR) may be seen in close contact with dental restoration or provision of a denture. These lichenoid reactions are usually the result of a contact sensitivity or irritant contact response (Type 4 immune reaction) to an amalgam or composite resin dental restoration or a denture component in close proximity to the oral mucosa (3).

Lichenoid reactions (LR) should, by definition, resolve after the causing agent(s) are removed.



Figure 9: Lichenoid reaction (LR) on buccal mucosa. Hypersensitivity reaction to amalgam (type IV immune reaction). Removal of amalgam usually leads to healing of the lesion.

From: www.hindawi.com.

Traditionally Both CD8+ and CD4+ effector T lymphocytes, including Th1, Th2 and Th17 are thought to be the predominant cell types in OLP (5). We observed (Koren et al, IOB seminar 2005), that regulatory T-cells (CD4+CD25+FoxP3+ Treg) make up a large part of the lymphocyte infiltration in OLP, an observation confirmed by others (10). Thus the modulating role of Tregs in this chronic condition has become a focus of research interest (10).

T cell maturation is regulated by professional dendritic cells (DC) which play an important role in initiating and controlling the immunologic activity of this disease (6,7). But even the DC needs to be differentiated in order to function as immune regulators.

In 2008 Singh et al., reported that a self peptide derived from ApoE had the ability to differentiate murine and human monocytes to become dendritic cells which functioned as immune modulators and suppressed inflammation (5).

By using global gene expression studies (microarray) of OLP lesions we had revealed that ApoE was 8-12 times more expressed in diseased OLP mucosa compared to none, or less diseased areas.

Thus, could increased ApoE expression explain some of the immune regulatory phenotypes observed in OLP?

Background and Theory

Dendritic Cells are professional antigen presenting cells (APC). They can induce, sustain and modulate immune responses in humans (14). DC's engulf, process and present antigens on MHC-II molecules to different subsets of CD4+ T-helper cells (see figure 1). Dendritic cells have a modulating effect on cytotoxic CD8+ T lymphocytes and B cells among others (14). DC's of lymphoid and myeloid origin are found in both humans and mice

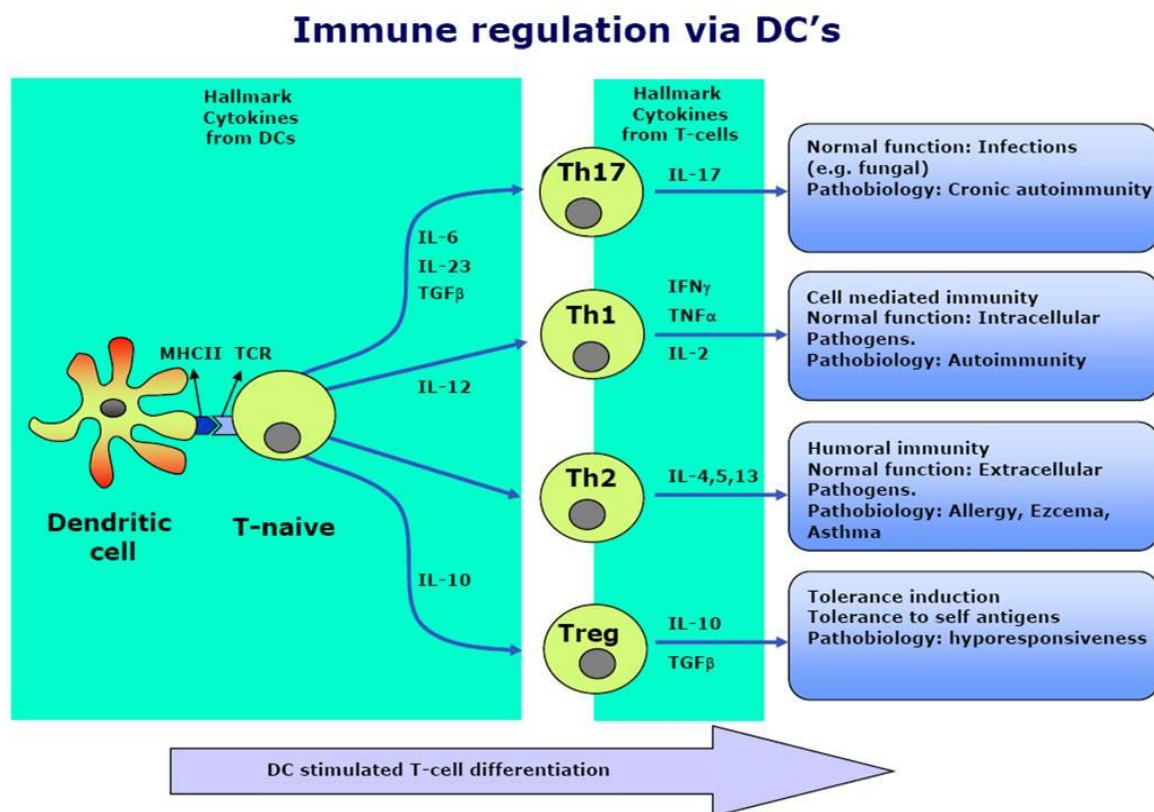


Figure 11: Immune regulation via Dendritic Cells (11). Th17 has mostly extracellular functions. Th1 cells are predominantly against intracellular virus and bacteria. Th2 is primarily effective against parasites and is connected to Eosinophilic reactions. The main role of Treg is suppression of inflammation.

T lymphocytes are cells of the lymphoid pathway that mature in thymus. They play a vital role in the cell-mediated immune system. All T cells express T cell receptor (TCR) on the surface. Several different types of T cells exist, including CD4⁺ T-helper cells (Th17, Th1, Th2 and Treg), CD8⁺ cytotoxic T-cells, T memory cells, regulatory T-cells (Tregs) and natural killer T cells (NKT).

Tregs are a subpopulation of T cells. They have properties that enable them to modulate immune responses. The most known of these cells are CD4⁺ T cells that co-express CD25 and Foxp3 (CD4⁺CD25⁺Foxp3⁺ T cells) (5, 16). CD25 is the alpha chain of the IL-2 receptor for IL-2. There are two major types of CD4⁺CD25⁺ T-cells. Natural occurring Treg (nTreg) derive from the thymus whereas peripherally induced Tregs are named: iTreg (16). Foxp3 is a transcription factor and serves as a master regulator of the function and development of CD4⁺CD25⁺ regulatory T cells (10, 16). Mutations in the Foxp3 gene results in loss of function for both nTreg and iTreg (16). Many of the intra- and sub-epithelial T cells in OLP, express Foxp3 and CD25⁺ (10). This suggests that Treg play a key role in regulating the complex immune reaction in OLP. DC-induced Treg division requires IL-2. This is mediated by conventional CD4⁺ T cells via MHC-II interactions (20).

ApoE: Apolipoprotein E is a class of lipoprotein that has an important role in the metabolism of lipids. It is found in chylomicrons and on LDLs (low density lipoproteins). ApoE is synthesized and secreted by many tissues, primarily liver, brain, skin, and tissue macrophages throughout the body. In addition to the well established role as a lipid transport protein, ApoE has also a modulating function in immune responses with the ability to either inhibit or stimulate antigen- and mitogen-induced T lymphocyte activation and proliferation (9, 12).

ApoE is located on chromosome 19, along with ApoC1 and ApoC2. It consists of 284 base pairs, with 3 different isoforms: ApoE2, ApoE3 and ApoE4 (13).

E2 is associated with hyperlipidemia type III, E3 is the most common and thus considered the normal variant and E4 is associated with increased risk of atherosclerosis and Alzheimer's disease. ApoE has also been attributed to immune regulation (9).

The allelic forms differ from each other only by amino acid substitutions at positions 112 and 158. [7] The E2 allele has a Cys at positions 112 and 158 in

the receptor-binding region of ApoE. The E3 allele is Cys-112 and Arg-158. The ApoE E4 allele is Arg at both positions (9,13)

Macrophages are among the cells that express high levels of ApoE (14). Production takes place in brain, spleen, liver, kidney, and muscle tissue (21).

Theory:

Macrophage production of ApoE may stimulate dendritic cells to become tolerogenic. Leading to a potent stimulation of different subsets of T-lymphocytes and thus induce local Treg differentiation.

Materials and methods

Microarray

Microarray is a collection of microscopic DNA spots attached to a solid surface (e.g. glass or silicon chip). DNA microarray is used to measure the expression levels of large numbers of genes in one test. Each DNA spot contains a specific DNA sequence, known as probes. These can be a short section of a gene or other DNA. Probe-target hybridization is usually detected and quantified by to determine relative abundance of nucleic acid sequences, and thus the gene expression profile, in the target tissue.

An array contains thousands of these DNA sequences. This makes it possible to perform many genetic tests at the same time.

DNA microarrays can be used to measure changes in gene expression levels, detection of single nucleotide polymorphisms (SNPs), or to genotype any targeted sequence.

The core principle of microarray is hybridization between two DNA strands. Complementary nucleic acid sequences pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. After washing off of non-specific bonding sequences, only strongly paired strands will remain hybridized.

Fluorescently labeled target sequences that bind to a probe sequence generate a signal that depends on the hybridization conditions (such as temperature), and washing after hybridization. Total strength of the signal, from a spot (feature), depends upon the amount of target sample binding to the probes present on that spot.

Microarrays use relative quantification in which the intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position. This makes it possible to compare sick and healthy tissue.

Two-color microarrays or two-channel microarrays are typically hybridized with cDNA prepared from two samples to be compared (e.g. diseased tissue versus healthy tissue) and that are labeled with two different fluorophores (17). This technique was used in our study.

Fluorescent dyes commonly used for cDNA labeling include Cy3, which has a fluorescence emission wavelength of 570 nm (corresponding to the green part of the light spectrum), and Cy5 with a fluorescence emission wavelength of 670 nm (corresponding to the red part of the light spectrum).

The two Cy-labeled cDNA samples are mixed and hybridized to a single microarray that is then scanned in a microarray scanner to visualize fluorescence of the two fluorophores after excitation with a laser beam of a defined wavelength (see figure 3 and 4). Relative intensities of each fluorophore may then be used in ratio-based analysis to identify up-regulated and down-regulated genes (18).

Biopsies from two patients (LR33 and LR34) were stored on RNAlater (SIGMA R0901) to prevent RNA degradation of biological material subject to RNA extraction. Samples stored in RNAlater RNA Stabilization Reagent stabilize and protect cellular RNA from 1 day (37C) to 4 weeks (2-8C) (22).

These biopsies were later used for Microarray study, RT-PCR and IHC.

RNA was extracted with RNeasy Mini Kit (Qiagen cat. 74104) (column based RNA extraction).

DNA Array 900 Cy3TM/Cy5TM Kit (Genisphere cat. W500180) was used to make labeled cDNA with fluorescent dyes.

Cy-3/Cy-5 labeled cDNA was hybridized to micro array slides in a “Slidebooster” (Advalytix). This ensures correct temperature, moisture and mixing during hybridization.

Slides scanned in “ScanArray Lite” (Packard Bioscience).

The results were processed in J-express.

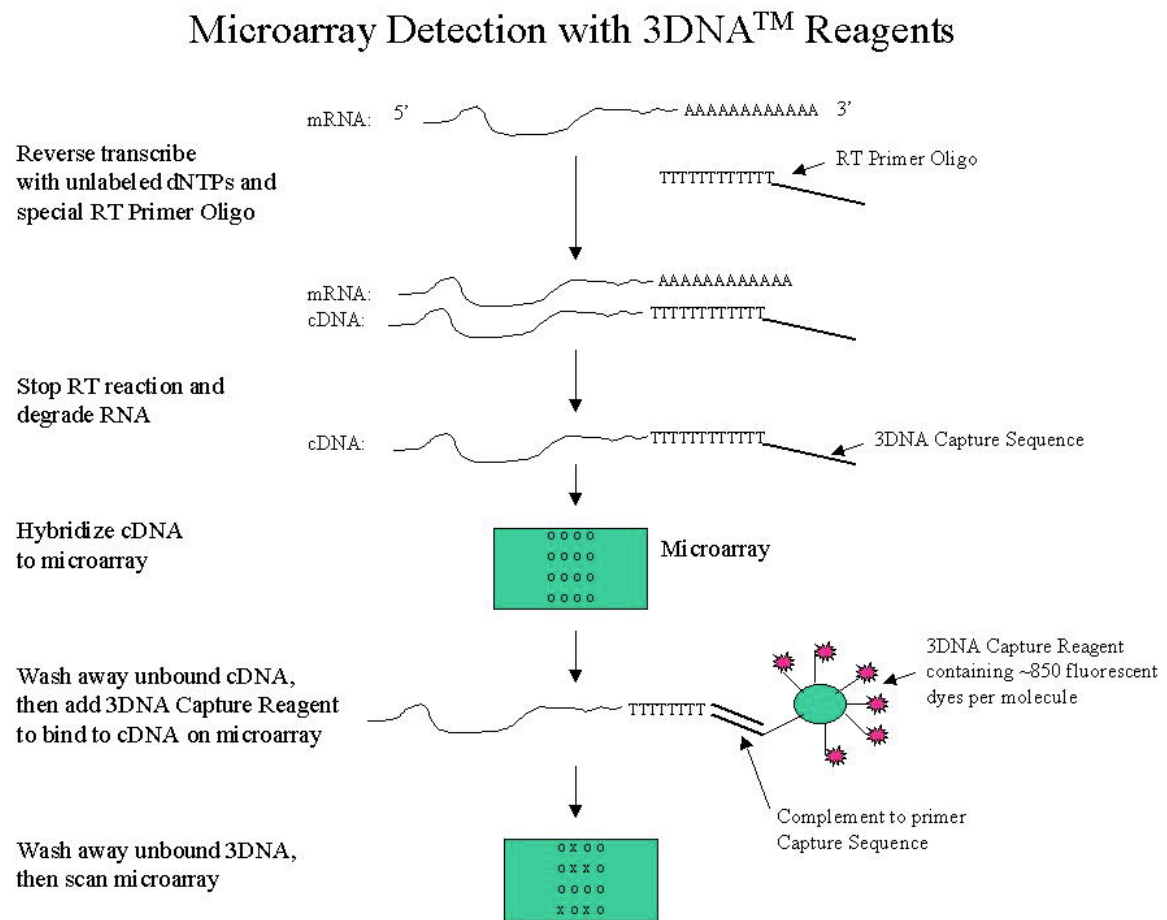


Figure 12: Microarray Detection with 3DNA Reagents (17)

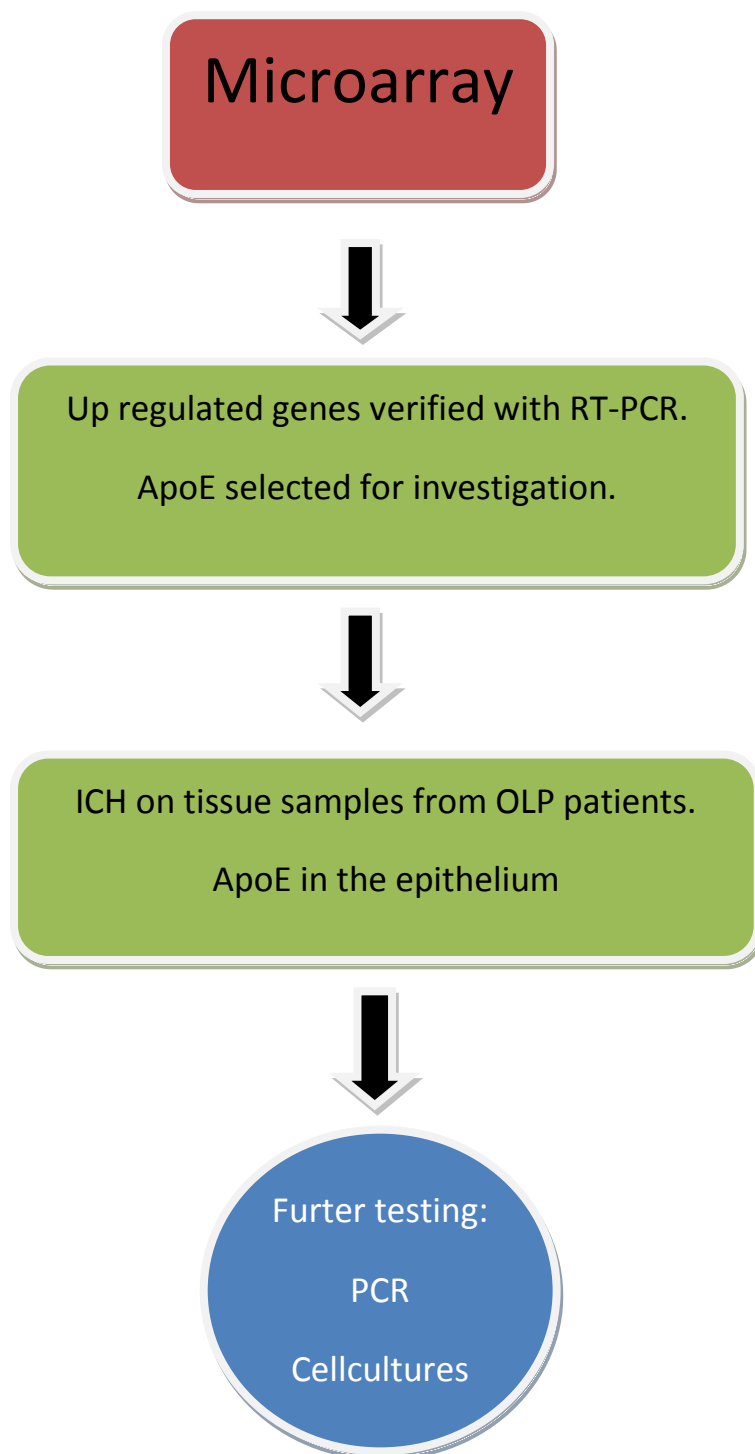


Figure 13: RNA extracted from biopsy is processed with microarray. Results revealed that ApoE was 8-14 times more expressed in tissue with high degree of inflammation compared to areas with lower degree of inflammation in the same patient. Results were confirmed with Rt-PCR. This gave a basis for further studies. Tissue samples taken from the same patients were tested with immunohistochemistry.

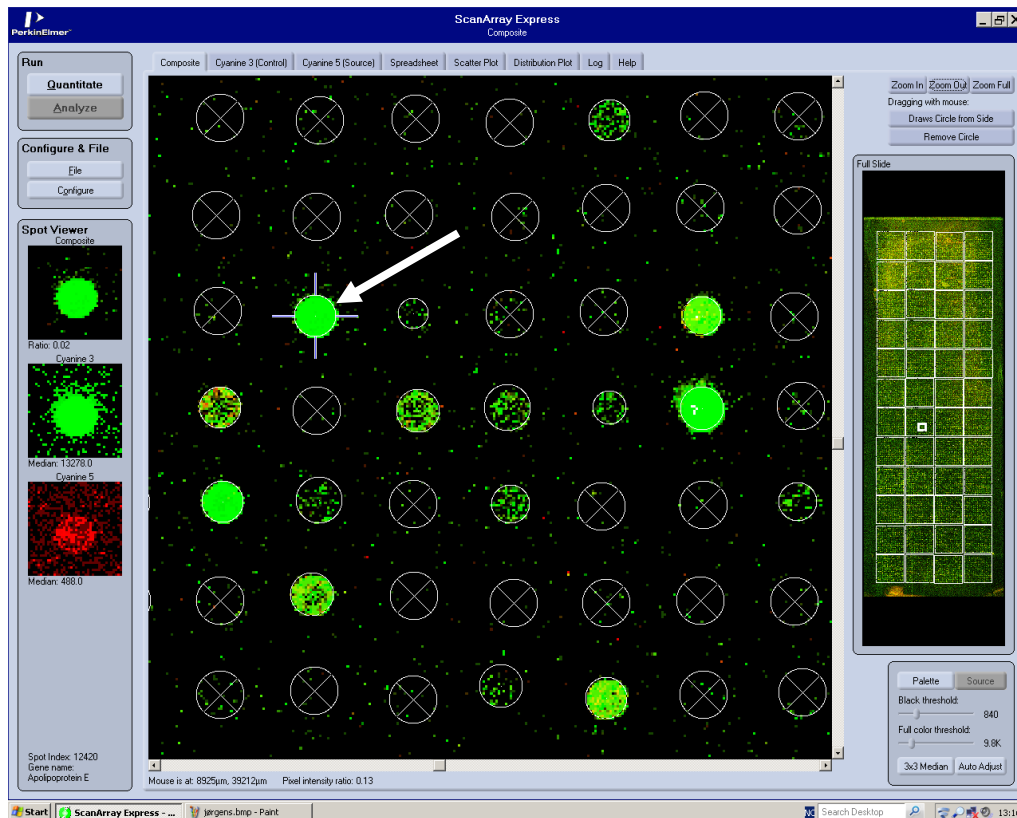


Figure 14: Microarray results showing high levels of ApoE expressed in OLP tissue indicated by the intense green spot (arrow).

Real Time Polymerase Chain Reaction

Real-time polymerase chain reaction is a method used for amplification and quantification of a targeted DNA molecule. The procedure follows the general principle of polymerase chain reaction (PCR). The amount of amplified DNA is detected in real time.

Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

Real-time PCR can be combined with reverse transcription to quantify mRNA and non-coding RNA in cells or tissues.

A DNA-binding dye binds to all double-stranded (ds) DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle. This allows DNA concentrations to be quantified.

In our study cDNA was synthesized with Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas cat. 1631). Using RealMasterMix (Qiagen cat. 0032 002.492) PCR machine MX 3005P (Agilent).

The following primer sequences were used in our study for RT-PCR:

APOE Forward primer: CTGCTCAGCTCCCAGGTC

APOE Reverse primer: TTGTTCTCCAGTTCCGATT

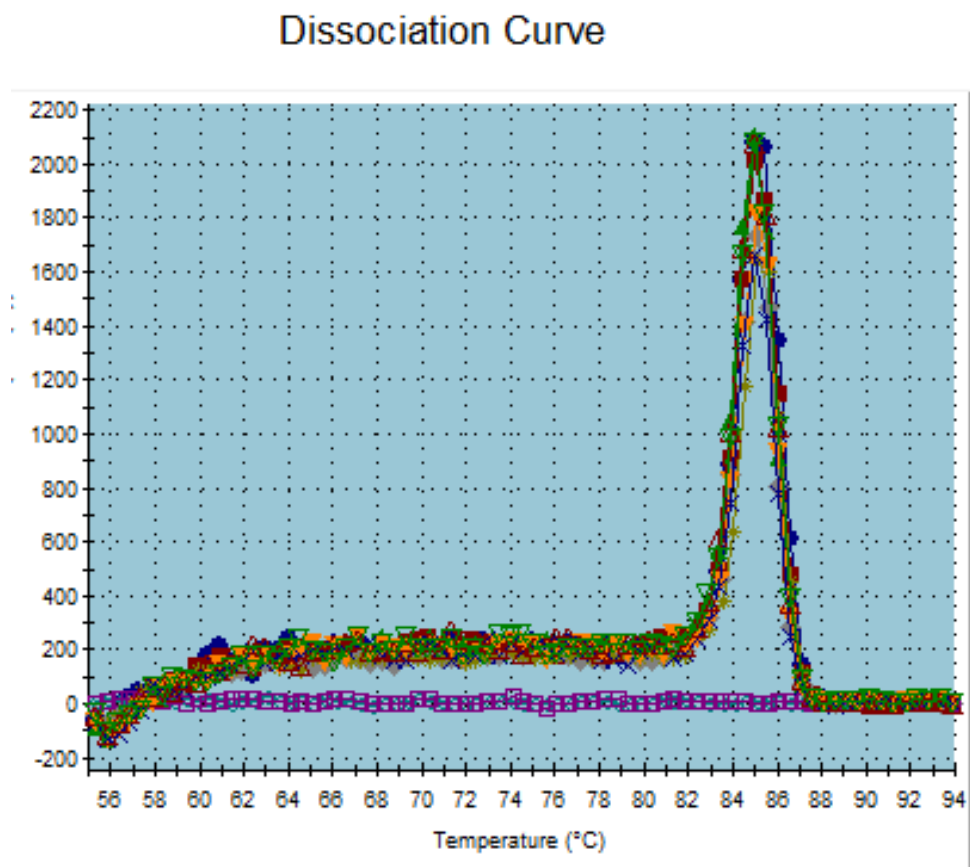


Figure 15: RT-Pcr dissociation curve. Also known as melting curve analysis. Show the temperature where 50% of DNA strands separate into single strands. It is used to determine the purity of the amplification. Diagram with one clear peak as shown above indicates amplification without non-specific products or contamination. In addition gel electrophoresis was performed on the PCR to confirm the specificity of the primers.

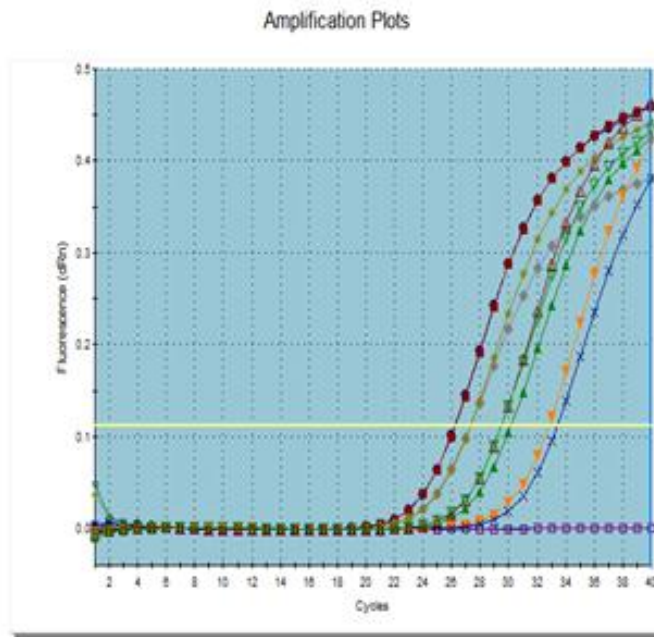


Figure 17: Amplification plots.

X-axis show number of cycles. Y-axis show fluorescence intensity (SYBR Green). Gene of interest ApoE from 6 samples. Samples with highest level of ApoE furthest to the left and lowest level to the right.

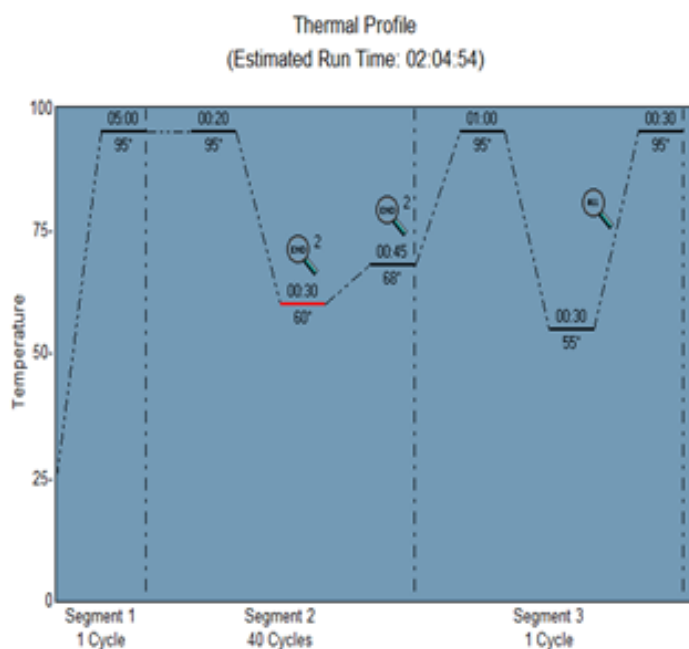


Figure 16: Thermal profile. Temperature changes in the Rt-PCR machine.

Segment 1: denaturation of DNA. Segment 2: amplification cycles with denaturation, annealing (primer binding) and extension. Cycle repeated 40 times. Segment 3: Dissociation curve to determine the purity of the

Immunohistochemistry (IHC)

Immunohistochemistry is the process of detecting antigens in cells of a tissue. The principle of this method is antibodies binding specifically to antigens in a specific biological tissue.

Visualizing an antibody-antigen interaction can be accomplished in different ways. Usually an antibody is conjugated to an enzyme, such as peroxidase, that can catalyze a color-producing reaction (see figure 5).

Alternatively, the antibody can also be tagged to a fluorophore, e.g. fluorescein or rhodamine.

The antibodies used can be polyclonal or monoclonal. Polyclonal antibodies are made by injecting animals with peptide Ag. A secondary immune response is stimulated and finally antibodies are isolated from whole serum. Polyclonal antibodies are a heterogeneous mix of antibodies that recognize several epitopes. Monoclonal antibodies show specificity for a single epitope and are therefore more specific to the target antigen than polyclonal antibodies.

Antibodies used in IHC testing are classified as primary or secondary reagents. Primary antibodies are raised against an antigen of interest and are typically unconjugated (unlabeled). Secondary antibodies are raised against immunoglobulins of the primary antibody species. The secondary antibody is usually conjugated to a linker molecule (Figure 12).

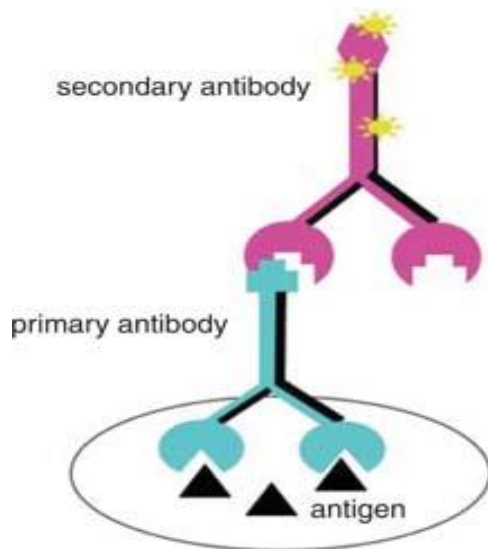


Figure 18: Primary and secondary antibody (18). Primary antibody binds to antigen epitop. Secondary antibody binds to a seat on the primary antibody. Secondary antibody is marked with fluorescent material making it possible to visualize.

Antigen	Producer	Host	Isotype
CD68, macrophage	Dako	Mouse	IgG1
HLA-DR	DB Biosciences	Mouse	IgG2a
CD86, B7-2	R&D	Mouse	IgG1
CD209 (DC-SIGN)	Beckman Coulter	Mouse	IgG1
Apolipoprotein E	Abcam	Rabbit	-
DEC-205/CD-205	R&D	Goat	IgG

Figure 19: List of antibodies used in our study.

“Axioplan 2” (Zeiss) fluorescence microscope was used for IHC analyses.

Cell culture

The two human monocytic cell lines THP-1 and U-937 were cultured in RPMI 1640 with 10% FCS (Fetal Calf Serum).

Immunofluorescence staining was also performed after cytopsin on stimulated THP1 and U937 cell lines (Ap1.B). The negative humanized control peptide was similar as the active humanized peptide but alanine was substituted for phenylalanine in position 11, called hEp1.N .

1 million cells were grown in 1ml culture media in a 24 well culture plate, stimulated with peptides for 24 and 48 hours prior to RNA extraction. Cytopsin was performed because the cellular “clumping” made flowcytometric analysis impossible. These cells were then subjected to IHC analysis.

Cell culture setup used in our study:

- 13 : Negative control, cell line U937 (no peptide) for 24 hrs
- 14: Cell line U937 incubated with humanized peptide hEp1.B for 24 hrs
- 15: Cell line U937 incubated with the humanized control peptide hEp1.N for 24 hrs
- 16: Negative control, cell line THP-1 incubated without peptide for 24 hours
- 17: Cell line THP-1 incubated with the humanized peptide hEp1.B for 24 hours
- 18: Cell line THP-1 incubated with the humanized control peptide hEp1.N for 24 hours
- 19: Cell line THP-1 incubated with the humanized peptide hEp1.B for 48 hours
- 20: color control

Flow cytometri was not performed because of the precipitation. Lack of single cells made it impossible to run flow cytometri.

To make a human analogue of Ep1.B, two amino acids were replaced. The peptide was synthesized by Thermo Fisher Scientific.

Human Ep1.B sequence (hEp1.B): AQQIRLQAEAFQAR.

Negative humanized control peptide hEp1.N sequence: AQQIRLQAEAAQAR.

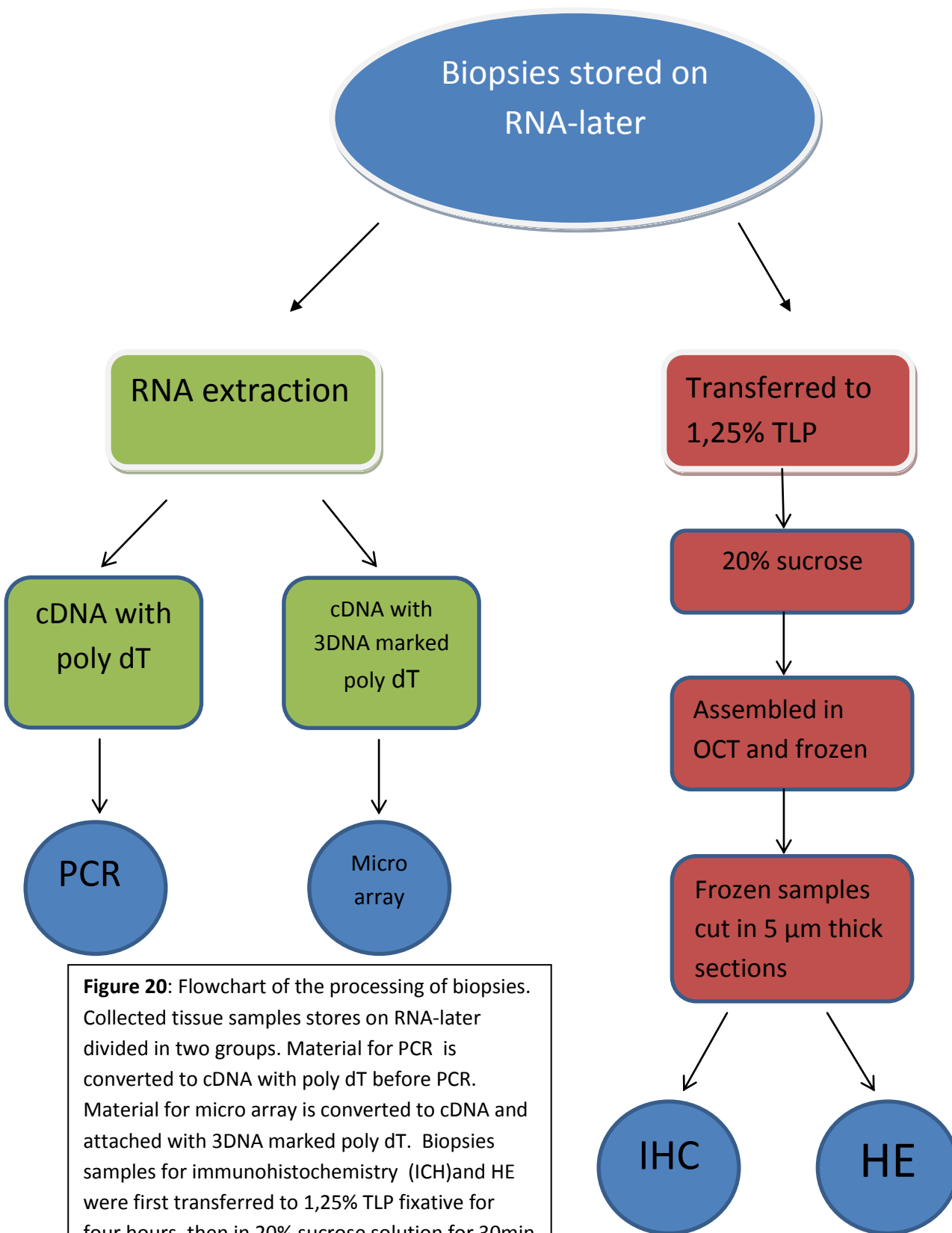


Figure 20: Flowchart of the processing of biopsies. Collected tissue samples stores on RNA-later divided in two groups. Material for PCR is converted to cDNA with poly dT before PCR. Material for micro array is converted to cDNA and attached with 3DNA marked poly dT. Biopsies samples for immunohistochemistry (ICH) and HE were first transferred to 1,25% TLP fixative for four hours, then in 20% sucrose solution for 30min before assembled in OCT, frozen and then cut in 5 μ m thick sections before ICH and HE testing could proceed.

Results

Previous work at our laboratory has shown an increase in regulatory T-lymphocytes in oral lichen planus (OLP) (10).

mRNA for ApoE is upregulated in OLP.

Preliminary IHC coloring showed that ApoE was present in the epithelium of both patients.

Microarray done on biopsies from 2 patients with OLP showed an increase of ApoE in sick tissue compared to tissue with less degree of inflammation in the same patients. Expression levels of ApoE were up regulated 8,8 times in sick tissue compared to tissue taken outside the lesion in patient LR33. In patient LR34 the expression level was increased over 14 times (figure 21). Even though this is a low number of patients, the results were consistent and gave us a basis for further investigation.

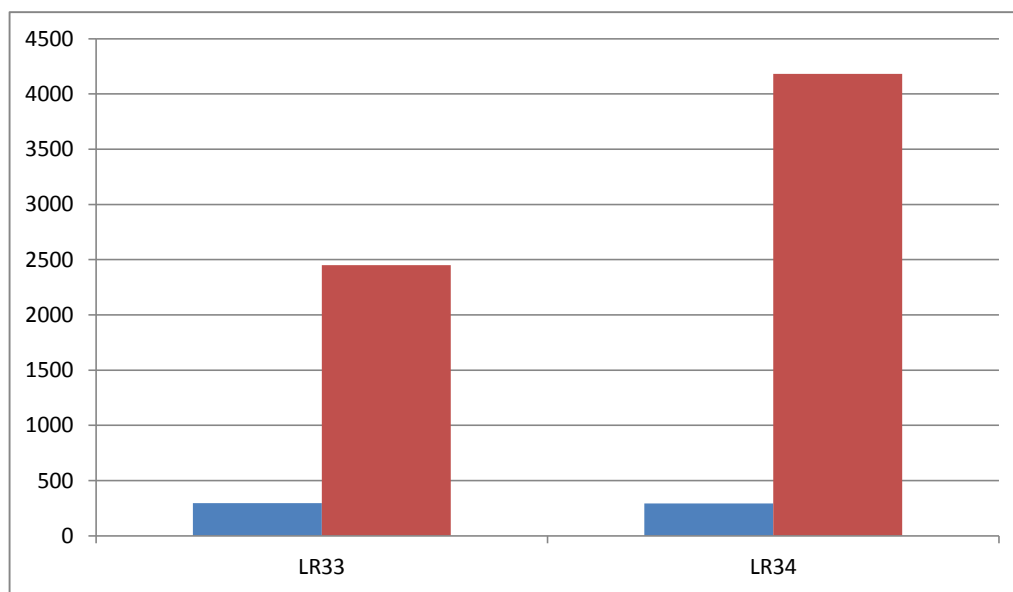


Figure 21: Micro array analysis of ApoE showing increased expression levels in lesions with oral lichen planus compared to control tissue. In patient LR33 the level of ApoE was increased 8.2 times. In patient LR34 the level was increased 14.31 times.

Real time PCR showed that ApoE was up regulated in OLP, confirming the previous results from our micro array. The results from PCR and micro array were very consistent.

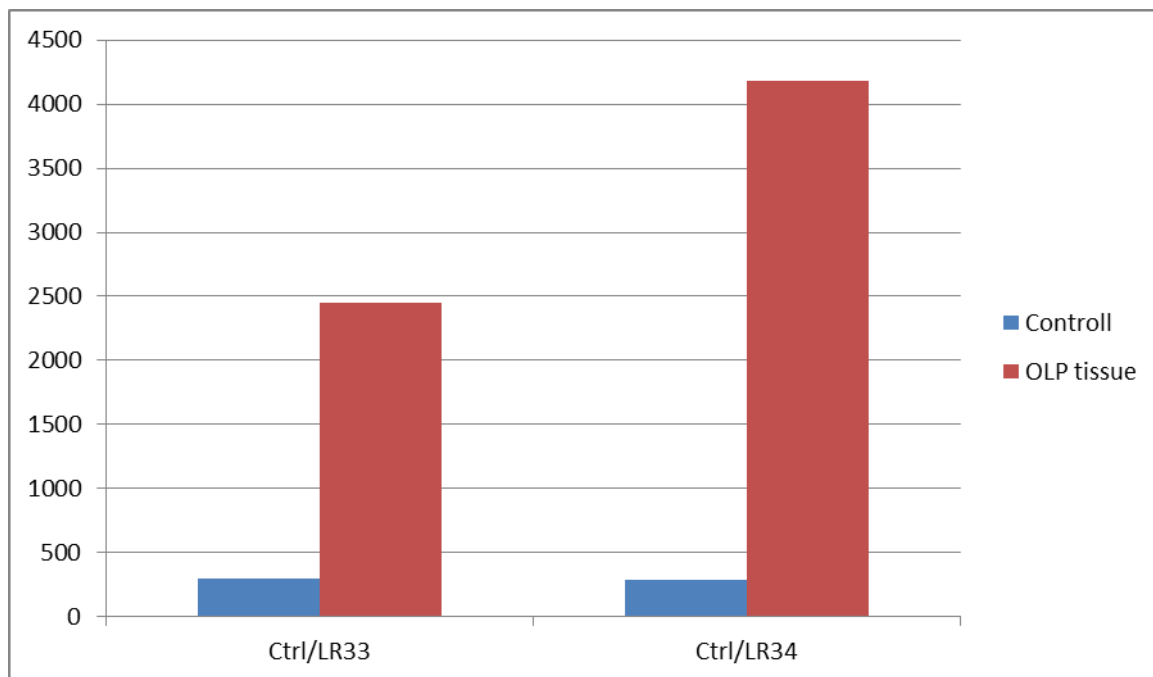


Figure 22: Real-time PCR analysis showing difference in levels of ApoE between tissue with low degree of and inflammation and tissue taken from area with high degree of inflammation in 2 patients. In patient LR33 the expression levels of ApoE was increased by 8.2 times in sick tissue. In LR34 the expression level was increased 14.32 times. This confirmed our microarray findings.

Biopsies from seven OLP-patients were cut in smaller pieces before RNA-extraction. APOE and CD3 expression where quantitated by real-time PCR. The CD3 expression where used as a measure of the density of the infiltrate. APOE mRNA where up-regulated in areas with denser infiltrate (Figure 23)

The three outliers with very high APOE expression were samples with low RNA yield and lower alpha-tubulin expression, suggesting that these were parts of the biopsies containing adipose tissue.

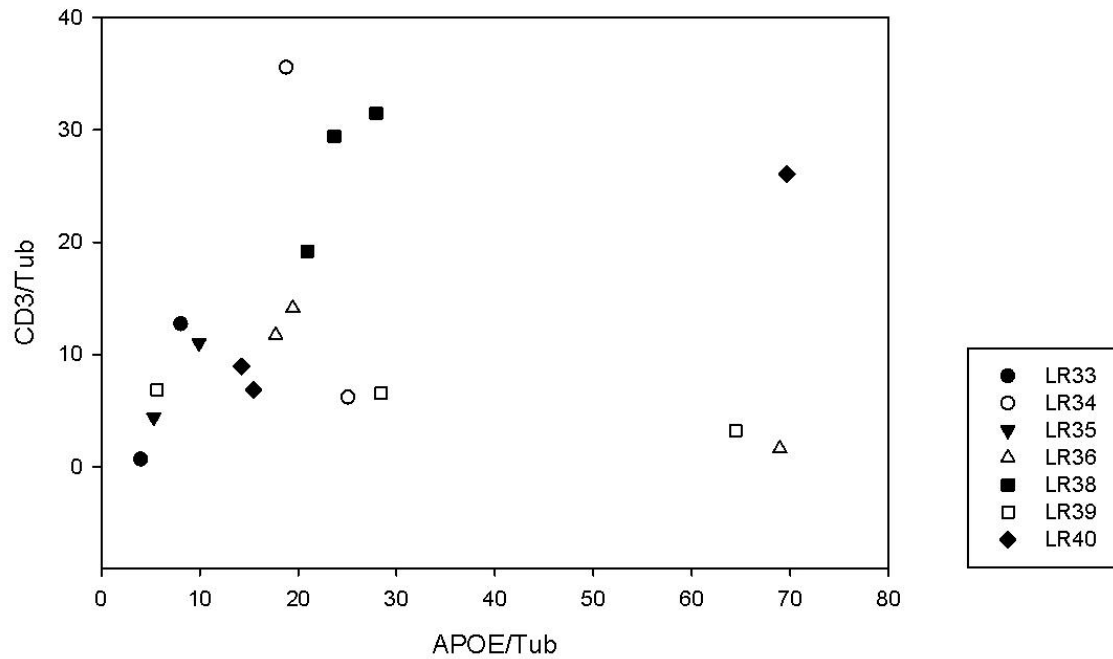


Figure 23: Real-time PCR on biopsies from 7 patients shows higher expression of APOE in areas with denser infiltrate (higher CD3).

hEp1B precipitate in culture media

The peptide was first added to the culture medium at a concentration of 200 µg/ml and vortexed. The peptide was in solution before adding 500 µl of cells. After some time, the peptide precipitated in the culture media. The same happened in media alone, both in serum free medium, and in medium supplemented with calf serum. The peptide precipitated in the serum-supplemented medium, only.

When changing the peptide to a human analog of the murine peptide, The two amino acids which were replaced in order to humanize the peptide, replaced one hydrophilic with a hydrophobic amino acids increasing the number of hydrophobic residues from 47 % to 50 %. 50% hydrophobic residues are about the limit for a water soluble peptide. However, the control peptide had similar 50% hydrophobic residues without any precipitating properties, arguing against this as the sole explanation for the observed precipitating.

hEp1B peptide induced cell aggregation but not DC-like morphology

Both U937 and THP-1 grow as semiadherent cells. When stimulated with the hEp1B peptide, first the adherent cells started to detach in sheets after 30

minutes, and then later on the cells aggregated in clusters. No dendritic morphology was seen.

Dendritic cells in Oral Lichen Planus

IHC was performed on biopsy tissue from OLP. Samples from small intestine and palatine tonsils were used for controls. Double staining was performed on all samples.

We used dendritic cell markers DEC-205 (CD205), CD11c, CD103, CD19, as well as Garp, ApoE, Foxp3 and LAR (see antibody list). DEC-205 is a cell surface receptor for CpG oligonucleotides. Primarily expressed on DCs, but also expressed on B cells, T cells and thymic epithelial cells. It is significantly up regulated in mature DCs. It plays an important role in uptake of extracellular proteins, which then are processed internally and subsequently presented on HLA class-II molecules.

The epithelium had a homogenous increased staining for ApoE, which was difficult to discriminate from background staining. There were many ApoE+ cells in the sub epithelial infiltrate, but none of these co-expressed CD11c or CD205 (DEC205).

This was in contrast to tonsillar tissue where almost all ApoE+ macrophages co-expressed CD11c+ cell, particularly in the germinal centers (tingle body macrophages).

DEC 205 was expressed by the basal keratinocytes particularly in less inflamed areas. Many DEC-205+ macrophage/dendritic cells were observed in the deeper subepithelial area, whereas CD11c + cells were located in the lamina propria.

DEC-205 was translocated in peptide stimulated cell cultures.

U937 cells stimulated in culture for 24 hours without peptide was weak CD11c+ and DEC205+, a few cells (~1%) were CD86+, none were DC Lamp+. Stimulating U937 cells with the active peptide hEp1.B for 24 hours revealed no increased expression of CD11c or DEC-205 compared to cells stimulated with the negative peptide, hEp1.N. THP-1 cells stimulated without peptide for 24 hours showed weak DC Lamp positivity but no DEC-205 expression. However, the majority of the cells were DEC-205+ when stimulated with hEp1.B for 24 hours, in contrast to those stimulated with the negative peptide hEp1.N, confirming the apparent up regulation of DEC-205 on the THP-1 cells. However

the humanized Ep1.B precipitated and induced cell aggregation, which may have activated the cells. Why this happened is not clear.

Discussion

Oral lichen planus is dominated by an intense mononuclear leukocyte infiltration composing of T cells and macrophages. We previously observed that 40% of the CD4⁺ T cells coexpressed Foxp3⁺, similar to regulatory T cells (Tregs). Thus these regulatory cells may control the intensity of the inflammation in OLP. By microarray based gene expression screening, we observed a peculiar strong mRNA increase for lipoprotein ApoE in OLP. ApoE belongs to a family of lipoproteins which predominantly have been implemented in fat metabolism. However in 2008 Singh *et al.* reported that a self-peptide derived from Apolipoprotein E stimulated monocytes to become dendritic cells with immunomodulating properties. Although Singh *et al.*, used a murine derived self-peptide sequence and showed that the murine monocyte cell line (PU5-1.8) were stimulated to become dendritic cells, it also worked on the human monocyte cell line (U937) (5). The self-peptide, Ep1.B, down regulated T cell proliferation and IFN- γ production and stimulated IL-10 secretion in immunized mice. Thus, it functioned as an immune regulator and suppressed the inflammatory process. Singh *et al.*, observed, moreover, that the Ep1.B peptide induced the monocyte cell lines to up regulate the DC-specific markers CD83, CD80 /CD86, CD11c and DEC-205.

The receptor for this self-peptide was not identified, but Toll like receptor 4 (the LPS receptor) and MHC-class II were excluded. The authors did not discuss how the murine self-peptide could exert its effect on human cells, as surface receptors do not tend to be so well conserved. The human version of this peptide differs with 2 amino acids, so we decided to use the human homolog for the self-peptide to stimulate human monocyte cell lines. Shifting the two amino acids from murine sequence to human sequence made the peptide somewhat less hydrophilic. Adding the humanized, active hEp1.B peptide to the culture media resulted in strong precipitation reactions, apparently making salt bridges as the precipitate was far bigger than could be explained by the concentration of the peptide alone (0.2 % solution). Moreover, the cells became attached to each other and formed large clusters which could not be resolved. This was only visible with the humanized hEp1.B peptide and not with the humanized control

peptide, which had similar hydrophilic properties. The cell clusters stayed together for more than 48 hours and made it impossible to perform flowcytometric analysis on stimulated cells. Thus we were forced to perform cytospin based immunofluorescence examination of the stimulated cells, instead of flow cytometric analysis which would have been preferable

The precipitation reaction was a surprise and made us wonder whether the concentration of the peptide was too high. However, Singh et al., used the peptide in 100 µg/ml peptide, which equals 61,3 µM (µmol/liter) which equals a 0,2% solution of the whole ApoE protein. This may not be too far from the local concentration during peptide release. However the reactions points to a possible non-specific protein-peptide-salt interaction as revealed by the strong precipitating reaction observed in both serum containing and serum free cell culture media.

Such non-specific protein-binding properties may also explain the induction of monocytes to dendritic cells in the murine system as the morphological changes was observed after 10 min (8). The peptide mimics, in this respect, the activation properties that lectins have on T cell activation.

We did not use the murine peptide. It is, therefore, unknown whether the precipitating effect of the humanized peptide was specific, or whether it also appeared with the murine peptide. The control peptide did not precipitate. Moreover, we performed several dilution experiments to prevent the precipitations, but none were successful. This reaction needs, however, clearly further investigation.

The peptide induced, nevertheless, increased expression of DEC-205 on the THP-1 cells, but not on the human U937 cells Singh et al., used. The immunofluorescence staining on the stimulated cells showed an increased surface expression of DEC-205, suggesting an increased translocation of intracellular DEC-205 after peptide stimulation. DEC-205 is known to be stored intracellular and may be translocated to the surface upon activation. This would explain Singh et al, results. However, we cannot explain why the U937 cells did not respond on the humanized hEp1.B peptide, in contrast to what Singh et al reported with the murine peptide. If similar self peptides derived from ApoE has similar effect on human monocytes it must be the human peptide sequence that matter. However, as long as we have not isolated the human self peptide, it

cannot be excluded that similar phenomenon exists in the human system, but that the self peptide is derived from other part of human ApoE. Only further studies may reveal whether such self peptides exist in human and whether these self-peptides has similar immunomodulation effect on human cells as reported by Sing *et al.*

Conclusion

Our study showed that Apolipoprotein (ApoE) is up regulated in oral lichen planus. It is however unclear if this up regulation play a role in the differentiation of monocytic cell lines to functional dendritic cells. Only membrane expression of CD205 was increased on THP-1 after incubation with the humanized hEp.1B. However, the humanized peptide precipitated in the cell culture media and induced cell adherence, which was not reported when the murine version of the self peptide was used. However one need to do the exact same setup as Singh *et al.*, used to examine if this response is selective for the humanized hEp.1B peptide.

ApoE is nevertheless increased in oral lichen planus, but our data do not support that self peptides from ApoE induce monocytes to differentiate into tolerogenic dendritic cells involved in the increased lesional density of Tregs in OLP.

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